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Abstract

The *in vitro* effect of a *Yucca schidigera* extract and tannins from chestnut wood on composition and metabolic activity of canine and feline faecal microbiota was evaluated. Four treatments were carried out: control diet (Group CTRL), chestnut tannins (Group CT), *Y. schidigera* extract (Group YSE) and Group CT+YSE. The YSE was added to canine and feline faecal cultures at 0.1 g/l, while CT were added at 0.3 g/l for a 24 h incubation. A total of 130 volatile compounds were detected by means of HS-SPME-GC/MS analyses. Several changes in the metabolite profiles of fermentation fluids were found including a decrease of alcohols (- 19%) and esters (- 42%) in feline and canine inoculum, respectively, due to the antibacterial properties of

tannins. In canine inoculum, after 6 h, Group YSE+CT resulted in lower cadaverine concentrations (- 37%), while ammonia (- 4%) and quinolone (- 27%) were reduced by Group CT. After 24 h, the presence of CT resulted in a decrease of sulphur compounds, such as dimethyl sulphide (- 69%) and dimethyl disulphide (- 20%). In feline faecal cultures, after 6 h, Group CT lowered the amount of indole (- 48%), whereas Group YSE tended to decrease trimethylamine levels (- 16%). Both in canine and feline inoculum, Group CT and, to a minor extent, Group YSE affected volatile fatty acids patterns. In canine faecal cultures, tannins exerted an inhibitory effect on *E. coli* population (- 0.45 log10 numbers of DNA copies/ml), while enterococci were increased (+ 2.06 log10 numbers of DNA copies/ml) by Group YSE. The results from the present study show that *Y. schidigera* extract and tannins from chestnut wood exert different effects on the composition and metabolism of canine and feline faecal microbiota. In particular, the supplementation of *Y. schidigera* and tannins to diets for dogs and cats may be beneficial due to the reduction of some potentially toxic volatile metabolites.

Keywords: dogs; cats; intestinal microorganisms; *Yucca schidigera*; tannins; volatile compounds.

Abbreviations: ADF, acid detergent fibre; ADL, acid detergent lignin; CT, tannins from chestnut wood; DM, dry matter; FOS, fructo-oligosaccharides; GC/MS, gas chromatography–mass spectrometry; NDF, neutral detergent fibre; PCA, principal components analysis; HS-SPME, headspace solid-phase microextraction; VFA, volatile fatty acids; VOCs, volatile organic compounds; YSE, *Yucca schidigera* extract.

1. Introduction

The canine and feline gastrointestinal tract is inhabited by a variety of complex microbial communities that play a fundamental role in maintaining the nutritional and health status of the host. Diet is recognized as one of the major factors driving the composition and metabolism of the gut microbiota (Russell et al. 2013). While many studies have highlighted the beneficial effects deriving from the dietary inclusion of non-digestible carbohydrates and prebiotics in dogs and cats (Pinna and Biagi 2014; Rochus et al. 2014), little is known about feeding functional food components to these animals.

Yucca schidigera Roezl ex Ortgies and tannin extracts are naturally occurring plant substances that have been widely investigated in swine and poultry production as potential phytochemical compounds for reducing odour and ammonia emissions (Windisch et al. 2008; Biagi et al. 2010).

Y. schidigera is known to have antimicrobial (Wang et al. 2000), antiprotozoal (McAllister et al. 2001), and antifungal (Miyakoshi et al. 2000) activities. The biological effects of *Y. schidigera* have been attributed to its high content of steroidal saponin fraction (Patra and Saxena 2009); however, Duffy et al. (2001) reported that the observed effects were also due to the non-saponin fraction.

Tannins are natural polyphenolic compounds that can be chemically divided into condensed and hydrolyzable tannins, whose structural diversity influences their metabolism and bioavailability (Aura 2008). Tannins extracted from chestnut (*Castanea sativa* Miller) wood are characterized by the presence of hydrolyzable tannins, which are known to act as effective microbiota modulatory agents in poultry (Jamroz et al. 2009), swine (Biagi et al. 2010), and ruminants (Hassanat and Benchaar 2013). While the administration of *Y. schidigera* to dogs and cats resulted in lower faecal odour (Lowe et al. 1997; Lowe and Kershaw 1997; Giffard et al. 2001), to the

best of our knowledge, there are no studies on the influence of tannins on the canine and feline intestinal ecosystems.

Considering that very little is known about the effects of *Y. schidigera* and tannin extracts in dogs and cats, the aim of the present study was to evaluate *in vitro* the effect of these extracts on the composition and activity of canine and feline faecal microbiota. We hypothesized that adding *Y. schidigera* and tannin to canine and feline faecal inocula may reduce the presence of potential pathogenic bacteria and toxic compounds.

2. Material and methods

The current study was carried out at the Laboratory of Animal Production of the Department of Veterinary Medical Sciences, University of Bologna, Italy.

2.1 Canine experiment

Five healthy adult dogs (mixed breed; average BW 18.0 kg; age 4 to 6 year) were fed the same commercial dry diet for adult dogs (Effeffe Pet Food S.p.A., Pieve di Porto Morone, Italy), for 4 week before collection of fresh faecal samples. The diet contained the following ingredients: dried poultry meat, rice and other cereals, oils and fat, dried eggs by-products, vitamins, and minerals. The macronutrient composition of the diet (as fed) was the following: water 53 g/kg, crude protein 229 g/kg, ether extract 154 g/kg, crude ash 69 g/kg, starch 370 g/kg, crude fibre 16 g/kg, neutral detergent fibre (NDF) 137 g/kg, acid detergent fibre (ADF) 90 g/kg, and acid detergent lignin (ADL) 43 g/kg. A sample of fresh faeces was collected from each dog immediately after excretion, pooled, and suspended at 10 g/l in prereduced Wilkins Chalgren anaerobe broth. The faecal suspension was used to inoculate (100 ml/l) a previously warmed (39 °C) and prereduced medium prepared according to Sunvold et al. (1995). Five 30-ml

bottles (each bottle containing 21 ml of faecal culture) were set up per treatment. The same dry food that was fed to the dogs used as faecal donors, was digested in triplicate using the 2-step procedure proposed by Biagi et al. (2016). After *in vitro* digestion, the undigested fraction was dried at 70 °C until a constant dry weight was obtained (19.3 g of undigested residue were obtained from 100 g of food dry matter (DM)) and its chemical composition was the following: crude protein 169 g/kg, ether extract 100 g/kg, starch 17 g/kg, crude ash 238 g/kg, crude fibre 76 g/kg, NDF 313 g/kg, ADF 162 g/kg, and ADL 59 g/kg.

Four treatments were carried out: 1) control diet (CTRL) with no addition of substrates, 2) group *Yucca schidigera* extract (group YSE), Syntonise (saponins content 10.5%, Sintofarm S.p.A., Guastalla, Italy), 3) group chestnut tannins (group CT), Farmatan 75 (tannins from chestnut wood, tannic acids 75.6%, Sintofarm S.p.A., Guastalla, Italy), 4) group YSE+CT. The YSE was added at a final concentration of 0.1 g/l, while CT was added at a final concentration of 0.3 g/l. The bottles also contained the *in vitro* digested commercial dry food for dogs at 20 g/l. These concentrations should reflect the amount of *Y. schidigera* and tannins extracts that reach the hindgut when they are included in a commercial extruded food for dogs (with a digestibility of approximately 80%) at a concentration of 1.0 and 3.0 g/kg, respectively. In each study, five bottles were prepared without any experimental substrate and with no addition of the digested food as a negative control. The pH of faecal cultures was adjusted to 6.7; bottles were sealed and incubated for 24 h at 39 °C in an anaerobic cabinet (Anaerobic System; Forma Scientific Co., Marietta, OH; under an 85% N_2, 10% CO_2, and 5% H_2 atmosphere). Samples of fermentation fluid were collected from each bottle at 6 and 24 h for the determination of pH, ammonia, biogenic amines, volatile fatty acids (VFA), volatile compounds (VOCs), and for microbial analysis.

2.2 Feline experiment

Four healthy European shorthair female cats (average BW 4.0 kg; age 4 to 6 year) were fed the same commercial dry diet for adult cats (COOP Italia, Bologna, Italy), for 4 week before collection of fresh faecal samples. The diet contained the following ingredients: cereals, meat and meat by-products, vegetable by-products, fish by-products, protein plant extract, oils and fat, minerals, and vegetables. The macronutrient composition of the diet (as fed) was the following: water 63 g/kg, crude protein 277 g/kg, ether extract 94 g/kg, crude ash 98 g/kg, starch 397 g/kg, crude fibre 33 g/kg, NDF 258 g/kg, ADF 50 g/kg, and ADL 16 g/kg. The same dry food that was fed to the cats used as faecal donors was digested in triplicate (Biagi et al. 2016). After in vitro digestion of the diet fed to the cats, the undigested fraction was dried at 70 °C (23.6 g of undigested residue were obtained from 100 g of food DM) and its chemical composition was the following: crude protein 135 g/kg, ether extract 23 g/kg, starch 56 g/kg, crude ash 281 g/kg, NDF 322 g/kg, ADF 133 g/kg, and ADL 53 g/kg.

The method used in this experiment reflects the one adopted for the canine experiment.

2.3 Chemical analyses

Analyses of commercial dry food and digested diets were performed according to AOAC International standard methods (AOAC, 2000; method 950.46 for water, method 954.01 for crude protein, method 920.39 for ether extract, method 920.40 for starch, method 942.05 for crude ash, and method 962.09 for crude fibre). Fibre fractions were determined according to the procedure described by Van Soest et al. (1991), where NDF was assayed with a heat stable amylase and expressed inclusive of residual ash, ADF was expressed inclusive of residual ash, and lignin was determined by solubilization of cellulose with sulfuric acid. Ammonia was measured using a commercial kit (Urea/BUN – Color; BioSystems S.A., Spain). Volatile fatty acids

were analysed by HPLC. For the determination of biogenic amines, samples were diluted 1:5 with perchloric acid (0.3 M); biogenic amines were later separated by HPLC and quantified through fluorimetry.

2.4 Microbial analysis

A 1-ml portion of fermentation fluid was collected from each vessel and centrifuged at 4 °C for 5 min at 18000 *g*. The supernatant was removed and immediately frozen at – 80°C for further analysis. Bacterial genomic DNA was extracted from remaining pellet using the QIAamp Fast DNA Stool Mini-Kit (QIAGEN GmbH, Germany). Isolated DNA concentration (ng/µl) and purity were measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Template DNA was diluted to 50 ng/µl and stored at – 20 °C until further analysis. *Escherichia coli* (Malinen 2003), *Bifidobacterium* genus (Matsuki et al. 2002), *Lactobacillus genus* (Collier et al. 2003), and *Enterococcus genus* (Rinttilä et al. 2004) were quantified via qPCR using specific primers.

The qPCR assay was performed using a CFX96 Touch thermal cycler (Bio-Rad, USA). Amplification was performed in duplicate for each bacterial group within each sample. Briefly, the PCR reaction contained 7.5 µl 2X SensiFAST No-ROX PCR Master Mix (Bioline GmbH, Germany), 4.8 µL of nuclease-free water, 0.6 µl of each 10 pmol primer, and 1.5 µl of template DNA for a final reaction volume of 15 µl. The amplification cycle was as follows: initial denaturation at 95 °C for 2 min, 95 °C for 5 s, primer annealing at 55–61 °C for 10 s and 72 °C for 8 s. The cycle was repeated 40 times. A negative control (without the DNA template) was also run for each primer pair. Standard curves were constructed from eight 10-fold dilutions for *Escherichia coli*, *Bifidobacterium* genus, *Lactobacillus genus*, and *Enterococcus genus*. Cycle threshold (Ct) values were plotted against standard curves for quantification of the

target bacterial DNA from faecal inoculum. Melting curves were checked after amplification to ensure single product amplification of consistent melting temperature.

2.5 Determination of total volatile compounds (VOCs)

A 1-ml portion of fermentation fluid was collected from each vessel, placed in a 7-ml vial with PTFE/red rubber septa (Supelco, Bellefonte, PA, USA) and capped. Volatile compounds were extracted by headspace-solid phase microextraction (HS-SPME) using a fused-silica fibre (10 mm length) coated with a 50/30 mm thickness of divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) and determined by GC/MS (QP-2010 Plus, Shimadzu, Japan), interfaced with a computerized system for data acquisition (Software GC-MS Solution V. 2.5, Shimadzu, Japan). A RTX-WAX column (30 m, 0.25 mm i.d., 0.25 μm film thickness, Restek, USA) coated with a stationary phase of polyethylene glycol, was used. The SPME fibre was first conditioned by heating it in a GC injection port at 270 °C for 60 min; then, it was inserted into the sample vial through the septum and exposed to the headspace (10 min at 60 °C) of the previously conditioned sample in a water bath at 60 °C for 60 min. Thereafter, the fibre was withdrawn into the needle and transferred to the injection port of the GC/MS system. The SPME fibre was desorbed and maintained in the injection port at 240 °C for 10 min. The sample was injected in split mode at a 1:30 split ratio. Helium was used as the carrier gas at a constant flow rate of 1 mL/min and a linear velocity of 36.2 cm/s. The oven temperature was kept at 40 °C for 10 min, then raised to 200 °C at 3 °C/min, and finally increased to 230 °C at 10 °C/min; final temperature was held for 5 min. The total run time was 74.33 min. Both injector and interface temperatures were set at 240 °C. The ion source temperature was set at 200 °C. The filament emission current was 70 eV. A mass range from 40 to 250 *m/z* was scanned at a rate of 0.25 scans/s. The acquisition and integration modes were Full Scan (TIC)

and Single Ion Monitoring (SIM), respectively. Compounds were identified by comparing their mass spectra with those contained in the NIST08 (National Institute of Standards and Technology, Gaithersburg) library and those reported in a previous study (Francioso et al. 2010). In order to control and prevent eventual environmental contamination, a blank injection of fibre and vials was carried out daily.

2.6 Statistical analyses

Data were analysed by 2-way ANOVA, with group YSE and group CT as the main effects; the Newman-Keuls test was used as the post hoc test. Five bottles (n = 5) were set up per treatment; each bottle represented an independent replicate. Differences were considered statistically significant at $p < 0.05$. The principal components analysis (PCA) was used to better understand the data variability. Statistical analyses were performed using Statistica 10.0 software (Stat Soft Italia, Italy).

3. Results

3.1 Chemical and microbial analyses

3.1.1 Canine experiment

pH values and concentration of ammonia and biogenic amines after 6 and 24 h of incubation with canine faecal inoculum are shown in Table 1. The pH values at 6 h were unaffected by treatments ($p > 0.05$), while, after 24 h of incubation, pH was significantly higher ($p < 0.001$) in vessels containing group CT. After 6 h of fermentation, ammonia was lowered ($p < 0.05$) by group CT. At 6 h, in the group CT+YSE vessels, the concentration of cadaverine was reduced ($p < 0.001$). After 24 h of incubation, group CT tended to lower the concentration of putrescine ($p = 0.088$) and decreased spermidine ($p = 0.010$). Finally, after 24 h of incubation, group YSE tended to reduce spermine concentrations ($p = 0.066$).

Volatile fatty acids concentrations at 6 and 24 h of fermentation with canine faecal inoculum are shown in Table 2. Only traces of n-valeric acid were detected (data not shown). Production of total VFA was decreased ($p < 0.004$) by group CT after 6 h, but not after 24 h of incubation. After 6 h of incubation, addition of group CT to the inocula resulted ($p < 0.05$) in lower concentrations of acetate, propionate and isobutyrate; the latter was decreased ($p = 0.009$) also by group YSE. After 24 h of incubation, group CT tended to reduce ($p = 0.083$) the concentrations of propionic acid.

The abundances of some bacterial populations in canine faecal inoculum are presented in Table 3. After 6 h, group YSE resulted in an increase ($p = 0.035$) of the presence of *Enterococcus* spp., whereas *E. coli* abundances were lowered by group CT ($p = 0.009$). After 24 h of incubation, the abundances of the assessed microbial populations were not affected by treatments ($p > 0.05$), but group YSE tended to reduce the presence of lactobacilli ($p = 0.053$). Bifidobacteria were inconsistently detected in canine faecal inoculum (data not shown). (Tables 1, 2 and 3 near here).

3.1.2 Feline experiment

The values of pH and concentration of ammonia and biogenic amines determined after 6 and 24 h of incubation with feline faecal inoculum, are shown in Table 4.

After 6 h of incubation, the pH level was reduced ($p = 0.021$) by group YSE, while, at 24 h, pH was higher ($p < 0.001$) in vessels containing group CT. Ammonia concentrations at 6 and 24 h were not affected by treatment ($p > 0.05$). Regarding the concentration of biogenic amines, cadaverine was lowered by group YSE at both 6 ($p = 0.017$) and 24 h (- 20%; $p < 0.001$), while an increase ($p = 0.040$) in spermine concentration was observed at 24 h in the group YSE vessels.

Concentrations of volatile fatty acids at 6 and 24 h of fermentation with feline faecal inoculum are shown in Table 5. After 6 h, concentration of total VFA tended to be lower for both group CT (p = 0.092) and group YSE (p = 0.052) treatments, while, at 24 h, total VFA concentration was significantly reduced (p = 0.002) by group CT. At 6 h, the presence of group YSE resulted in lower concentrations of acetic (p = 0.010) and valeric acids (p = 0.006). In bottles containing group CT, a reduction (p = 0.010) of the concentration of propionic acid was observed at 6 h, while a decrement of acetate (p = 0.044), propionate (p < 0.0001) and isovalerate (p = 0.039) was noted at 24 h. Similarly, at 24 h, group CT tended to reduce (p = 0.095) the concentration of butyrate.

Abundances of investigated microbial populations in the feline faecal inoculum are shown in Table 6. At 6 h, the presence of enterococci was reduced by group CT+YSE (p = 0.008). (Tables 4, 5 and 6 near here).

3.2 Volatile compounds (VOCs) detected by HS-SPME-GC/MS analysis

In all samples, a total of 130 different metabolites were identified, of which 36 were cat-related and 20 were dog-related (Supplemental Table 1). Most of VOCs that have been identified belong to the families of esters and alcohols; however, after 6 h of incubation, one of the most represented metabolites in feline and canine faecal inoculum was indole, which accounted for 47.1% and 39.7% of total VOCs area, respectively. After 24 h of fermentation, indole proportions decreased in both dog and cat inoculum (22.2% and 17.8% of total VOCs area, respectively). Other representative metabolites detected in both dog and cat vessels after 6 h of incubation, were ethanol, 1-propanol, 1-butanol, 3-methyl-1-butanol, and 1-hexanol (belonging to the family of alcohols), while phenol and dimethyl trisulphide were more predominant in dog faecal inoculum. After 24 h of fermentation, phenol was the most abundant metabolite detected in the dog faecal inoculum (32.9% of total VOCs area), followed

by indole, dimethyl trisulphide and dimethyl disulphide (22.2%, 11.2% and 6.7% of total VOCs area, respectively).

3.2.1 Canine experiment

Absolute area of VOCs obtained from the HS-SPME-GC/MS analysis of dog faecal inoculum is shown in Supplemental Table 2. After 6 h of incubation, hydrogen sulphide was higher ($p < 0.05$) in group CT vessels. Regarding aldehyde compounds, the addition of group CT resulted in higher amounts of caproaldehyde ($p = 0.024$) and nonaldehyde ($p = 0.017$) after 6 h of fermentation, and higher amounts of isovaleraldehyde at both 6 ($p < 0.001$) and 24 h ($p < 0.001$). Similarly, isovaleraldehyde amount was increased ($p < 0.05$) by group YSE at 24 h. On the contrary, after 6 h of incubation, a significant decrease ($p = 0.025$) of caproaldehyde was observed in YSE vessels. Quinoline was decreased by the presence of group CT at both 6 and 24 h ($p < 0.05$), while phenol was increased at 6 h by group CT ($p = 0.007$).

After 24 h of incubation, a decrease of the amount of dimethyl sulphide ($p = 0.058$) and dimethyl disulphide ($p < 0.05$) was observed in group CT batches; similarly, group CT decreased the amount of acetic acid butyl ester ($p < 0.001$), acetic acid propyl ester ($p = 0.003$) and total esters ($p = 0.006$).

3.2.2 Feline experiment

Absolute areas of the significantly different VOCs detected by HS-SPME-GC/MS analysis of feline faecal inoculum are shown in Supplemental Table 3. After 6 h of incubation, lower amounts of indole ($p < 0.001$), 1-butanol ($p = 0.008$), 1-butanol, 3-methyl ($p < 0.01$), 1-pentanol ($p = 0.019$) and total alcohols ($p = 0.035$), were observed in vessels containing group CT. Conversely, group CT increased ($p < 0.05$) the amount

of hydrogen sulphide after 6 h of incubation. Addition of YSE resulted in a decreasing trend in trimethylamine ($p = 0.069$) and ethanol ($p = 0.073$) amounts.

After 24 h, hydrogen sulphide amount was lower ($p < 0.05$) in batches where the combination CT+YSE had been added, while a reduction tendency ($p = 0.092$) of this metabolite was observed in group YSE vessels. Likewise, the addition of CT+YSE to the inocula tended to reduce total aldehydes ($p = 0.095$), 2-propanone ($p = 0.071$) and acetic acid ethyl ester ($p = 0.095$). After 24 h of incubation, a decrease ($p < 0.05$) of propanethioate-S-methyl and acetic acid 3-methylbutyl ester was observed in group CT vessels. On the contrary, the amount of *p*-cresol was increased significantly ($p = 0.002$) by group CT.

3.3 Principal Component Analysis (PCA)

3.3.1 PCA of canine experiment

To evaluate the effect of CT and YSE addition on VOCs formation and to define which compounds were the most representative of variance, the data were subjected to PCA. When samples of dog faecal inoculum were tested, the first two principal components reached 77.08% of variance (Supplemental Figure 1). The factor 1 explains 65.63% of variance; in particular, it is possible to observe that sulphur compounds, as well as acetic acid esters, alcohols, organic acids and quinolone, are inversely correlated to indole and carbon disulphide. In addition, a third cluster consisting mainly of aldehydes (isovaleraldehyde, caproaldehyde, nonanal, benzaldehyde and 2-methylbutyraldehyde) was more correlated to Factor 2 (11.45% of variance) (Figure 2). Considering the Biplot (Supplemental Figure 2), results at 6-h were well separated from the 24-h ones; in particular, the 6-h group CT assay was completely segregated from the other treatments, while all 24-h assays were plotted in the same cluster and

were mainly characterized by the presence of sulphur compounds, alcohols and acetic acid esters.

In order to better understand the variance of results, the PCA of all determined parameters from the canine experiment was carried out. As reported in Biplot (Figure 1), a total of 86.26% of variance was comprised in the first two principal components, of which PC1 explains 69.98% of total variance. In addition, it is possible to observe the presence of several clusters; in fact, PC2 evidences how lactobacilli, enterococci, total organic acids and 2-propanone were inversely correlated to the main aldehydes (e.g. caproaldehyde, benzaldehyde, etc.) and the 6-h control sample. On the other hand, the 24-h samples did not separate and were mainly characterized by the VOCs presence as reported above, which thus demonstrate a non-significant effect of group CT and group YSE after 24 h. (Figure 1 near here).

3.3.2 PCA of feline experiment

A PCA analysis of feline faecal VOCs was also carried out. Supplemental Figure 3 reports the component loading in the cat experiment; the first two principal components explained 81.78% of the total variance. In general, aldehydes, alcohols, organic acids and sulphur compounds were more correlated to Factor 1, which explained 67.26% of variance, whereas acetic acid esters, phenol and indole were correlated to Factor 2, which explained 14.52% of variance. In Supplemental Figure 4, a clear separation between 6-h and 24-h results is evidenced. After 6 h, group YSE and group CT treatments were mainly characterized by the presence of aldehydes (3-methylbenzaldehyde and isovaleraldehyde) and alcohols (3-methyl-1-butanol and ethanol), while VOCs at 24 h are mainly depicted by acetic acid esters, phenol, sulphur compounds, butyraldehyde and benzaldehyde. However, it might be pointed out that,

at 6 h, the combination YSE+CT was inversely correlated to the concentrations of indole, acetic acid propyl- and ethyl-esters and methyl propane thioate.

In addition, the PCA of all determined parameters was carried out (Figure 2). The 73.54% of total variance explained by the first two principal components, of which PC2 was more correlated to *Escherichia coli*, ethyl acetate and ketones. As shown in Figure 2, different clusters could be distinguished, in particular the 6-h samples were all gathered in the same cluster, characterized by aldehydes and ethanol, and inversely correlated to the presence of lactobacilli and organic acids. On the other hand, the 24-h samples (except for CTRL 24h) were mainly depicted by the presence of ammonia, short-chain alcohols and sulphur compounds. (Figure 2 near here).

4. Discussion

The results from the present *in vitro* study showed that *Y. schidigera* extract and chestnut tannins were able to exert some influence on the metabolism of canine and feline faecal microbiota.

4.1 pH values, SCFA production and bacterial protein catabolism

It is known that a low intestinal pH helps to improve the health of the gut mucosa by reducing unwanted bacteria and absorption of ammonia, while higher intestinal pH is thought to promote the growth of detrimental microorganisms (Zentek et al. 2013). When incubated with both canine and feline faecal inocula, tannins increased the pH of the fermentation fluid at 24 h, presumably as a consequence of the reduction of total VFA production. In fact, addition of CT to canine and feline slurries resulted in lower concentrations of acetate and propionate. Bravo et al. (1994) similarly reported that fermentation of tannic acid (1 g/l) with a rat caecal inoculum led to a decrease in acetic and propionic acid concentrations, while tannic acid at 2.5 g/l resulted in the total

suppression of acetic acid production, suggesting that tannic acid could be a specific inhibitor of acetate-producing bacteria. A decrease of SCFA concentrations in the colonic lumen may lead to mucosal atrophy as a consequence of a lack of energy for colonocytes (Machiels et al. 2014).

Compared with CTRL, group CT and group YSE significantly reduced the concentration of isobutyric acid in canine inocula, while group CT lowered the amount of isovalerate in feline inocula after 6 h. Isobutyric and isovaleric acid, as well as 2-methylbutyrate, mainly derive from the degradation of valine, isoleucine and leucine (Hughes et al. 2000), and are used as faecal markers for bacterial protein catabolism. In the first experiment, ammonia concentration, as well as spermidine concentration, were reduced by group CT, while the association CT+YSE decreased the amount of cadaverine. In presence of feline faecal inocula, group CT failed to decrease the amount of biogenic amines, even though group YSE reduced cadaverine but increased spermine concentration. Ammonia and biogenic amines are also products which derive from proteolytic reactions; in particular, biogenic amines are generally formed through the decarboxylation of specific free amino acids by microbial decarboxylases (Ku et al. 2013). The inhibitory effects of tannins on microbial proteolysis had already been observed by Biagi et al. (2010) in piglets (both *in vitro* and *in vivo*), and by Hassanat and Benchaar (2013) in presence of ruminal inoculum. *Yucca schidigera* is commonly used as a dietary additive in livestock and companion animals (Lowe et al. 1997; Lowe and Kershaw 1997) for the reduction of ammonia and faecal odour in animal excreta. Several mechanisms have been proposed to explain the effect of *Y. schidigera* extracts on ammonia concentrations in animal faeces, among which are the ability to inhibit the enzyme urease (Balog et al. 1994), to bind directly ammonia (McCrory and Hobbs 2001) and to inhibit specific bacterial strains (Patra and Saxena, 2009). However, in the present study, YSE failed to reduce ammonia concentrations. Similarly, ammonia

content of caecal material did not decrease in rats receiving sarsaponin (steroidal glycosides extracted from *Y. schidigera*) with the diet (120 mg/kg of diet; Preston et al., 1987).

4.2 Faecal microbiota

In this study, the addition of YSE to canine faecal inoculum resulted in an increase of enterococci but the same effect was not observed when group YSE was incubated in presence of the feline faecal inoculum. There is no evidence in the literature suggesting that *Y. schidigera* may act as a prebiotic so that, at present, we do not know if the increment of enterococci was the consequence of a direct effect of YSE on their growth or the result of the inhibition of enterococci antagonists by the saponins contained in the YSE (Killeen et al. 1998). Among the tested substrates, group CT resulted in a reduction of *E. coli* in the first experiment, while, in presence of feline faecal inoculum, *E. coli* abundances tended to be lower when CT was used in association with YSE. These results are not in agreement with those of Biagi et al. (2010), who reported a lack of antibacterial activity of chestnut tannins against coliforms, both *in vitro* and *in vivo*. It has been observed that yucca saponins may exhibit antibacterial effects on *E. coli* (Sen et al. 1998), but other authors did not report any bacteriostatic effect of *Y. schidigera* on pure cultures of *E. coli* (Killeen et al. 1998). However, Wang et al. (2000) showed that saponins from different sources, including *Y. schidigera*, are more effective against gram-positive than gram-negative bacteria (e.g. *E. coli*). The reduction of the presence of *E. coli* in the animals' intestine may be considered beneficial. However, when chestnut tannins were added to canine faecal inocula, minor effects were observed only after 6 h of incubation, which are probably without any physiological importance.

4.4 Volatile organic compounds in faecal inocula

To the best of our knowledge, our results provide the first characterization of VOCs profile of canine and feline faecal inocula by HS-SPME-GC/MS. A great variety of VOCs was found in the fermentation fluids. The addition of chestnut tannins to the canine faecal inocula led to an increase of hydrogen sulphide and a decrease of dimethyl sulphide and dimethyl disulphide, making the interpretation of these results very difficult, as both metabolites are produced from the catabolism of S-containing amino acids. Hydrogen sulphide is the main product of sulphate-reducing bacterial catabolism of cysteine and, when present at high concentrations, may inhibit the oxidation of butyrate by the colonic epithelium (Roediger et al. 1993). In general, hydrogen sulphide is methylated by the colonic epithelium to less harmful products, such as methanethiol and dimethyl sulphide (Weiseger et al. 1980). In the feline experiment, an increase of carbon sulphide and hydrogen sulphide was observed at 6 h in the vessels containing CT, while hydrogen sulphide was decreased at 24 h by group CT+YSE. In a previous *in vitro* study with dog faecal inoculum, the addition of *Y. schidigera* (0.17 g/l) resulted in lower (- 38%) hydrogen sulphide but did not affect total gas production (Giffard et al. 2001). In the study by Swanson et al. (2002), feeding dogs with a *Lactobacillus acidophilus* supplemented diet tended to increase dimethyl sulphide, methanethiol and hydrogen sulphide faecal concentrations. The ability of some species of *Lactobacillus* spp. to produce hydrogen sulphide has been reported by Arici et al. (2004). In the already cited study by Swanson et al. (2002), dogs fed fructo-oligosaccharides (FOS) at 2 g/d had the lowest faecal concentrations of S-compounds. Conversely, in the study by Hesta et al. (2005), the administration of FOS at 31 g/kg of diet (on DM basis) failed to reduce the faecal concentration of sulphur compounds in cats receiving a protein-restricted diet. Although in this study S-compounds accounted only for a small part of identified chemicals (1.40% and 2.65% of total

VOCs area after 6 h of incubation in presence of feline and canine faecal inocula, respectively), they are in general considered responsible for the distinctive bad odour of faeces. In fact, as reported by Hesta et al. (2005), S-containing components have an important impact on faecal odour, since six out of 10 compounds with the lowest odour detection threshold (degree of sensitivity of humans) contain sulphur.

While the utilization of CT led to contradictory results with regard to the concentrations of S-compounds, the experimental substrates used in this study were more effective in limiting the presence of N-compounds. In fact, group CT lowered the amount of quinoline (in presence of canine faecal inoculum) and indole (when incubated with feline faecal inoculum), while group YSE tended to lower the amount of trimethylamine in presence of cat faecal inoculum. Quinoline and indole are aromatic heterocyclic organic compounds; indole derives from microbial degradation of tryptophan in the colon (Shimada et al. 2013). In the past, indole was suspected to contribute to colon carcinogenesis, but Shimada et al. (2013) recently attributed to indole immunomodulatory and anti-inflammatory properties on intestinal epithelial cells. However, in a study by Nowak and Libudzisz (2006), indole showed negative effects on the *in vitro* viability of lactic acid bacteria. Trimethylamine is a tertiary amine derived from the bacterial catabolism of choline and, to a less extent, carnitine (Russell et al. 2013); trimethylamine can be further metabolized by gut microbiota to trimethylamine-N-oxide, a catabolite recently considered to be involved in human colorectal cancer pathogenesis (Xu et al. 2015). In the present study, trimethylamine levels tended to be reduced by group YSE.

The utilization of CT resulted in higher concentrations of phenol and *p*-cresol (4-methylphenol) in canine and feline faecal inocula, respectively. Phenol and *p*-cresol are recognized as potentially harmful by-products deriving from the bacterial catabolism of aromatic amino acids and are correlated with the development of cancer

in the human colon (Nowak and Libudzisz 2006). It has been shown that numerous mammals host bacteria that are able to degrade hydrolyzable tannins (Kohl et al. 2015). Hydrolyzable tannins are polyesters consisting of a carbohydrate core and phenolic acids (mainly gallic and ellagic acids), which are characterized by a phenolic ring and a carboxyl group. Based on our results, it may be supposed that the phenolic acids released from the tannins may be further metabolized to phenol and *p*-cresol by the gut microbiota. The slightly higher amount of phenol detected in vessels containing YSE and canine faecal inoculum may be the consequence of microbial degradation of polyphenolic compounds (resveratrol and yuccaols) that can be found in *Y. schidigera*. However, the microbial metabolism of resveratrol in the large intestine is still uncertain (Selma et al. 2009). Interestingly, the association CT+YSE resulted in a strong reduction of phenol concentrations, suggesting a synergistic effect of CT and YSE for which we do not have an explanation.

The observed reduction of the amounts of ketones and alcohols as a result of CT addition, seems to suggest a decreased microbial activity induced by tannins. In fact, ketones can derive from the microbial catabolism of fatty acids, while alcohols can be produced during sugar fermentation or reduction of organic acids by bacteria (de Lacy Costello et al. 2014).

Esters, the most numerous chemical class (36 identified compounds) in our samples, derive from the esterification of alcohols and fatty acids by the gut microbiota or by the esterase activity of the enterocytes (Garner et al. 2007). The lowering effect of group CT on esters abundance in the canine and, to a lesser extent, feline faecal inocula may be related, as observed by Daniel et al. (1991) in rats, to the inhibition of esterase activity by tannins.

Changes in the composition and abundances of VOCs in the headspace of vessels that were observed in this study can be directly related to the gut microbiota

metabolism and to the presence of the experimental substrates. Feeding *Y. schidigera* to dogs and cats has been demonstrated to reduce faecal malodour by sensory testing (Lowe and Kershaw 1997), while a concurrent study with dogs and cats showed changes in the faecal concentrations of several volatile components after *Y. schidigera* extract supplementation (Lowe et al. 1997). However, the authors did not find a direct correlation between the changes in the amounts of VOCs and the aroma amelioration. In fact, faecal odour is the resultant of absolute and relative concentrations of several compounds, the possible interactions among different chemicals and the impact of both texture and microstructure of faeces on release of odour compounds (Hesta et al. 2005; de Lacy Costello et al. 2014).

5. Conclusions

The results from the present study show that *Y. schidigera* extract and tannins from chestnut wood exert different effects on the composition and metabolism of canine and feline faecal microbiota. Although a decrease in malodorous compounds was observed, it is not possible to state whether or not the tested substrates lowered the inoculum odour. However, the dietary supplementation of *Y. schidigera* and tannins to dogs and cats may be beneficial due to the reduction of some potentially toxic volatile metabolites.

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Disclosure statement

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Table 1. pH values and mean concentrations (n = 5) of ammonia [mmol/l] and biogenic amines [µmol/l] after 6 and 24 h of an in vitro incubation of canine faecal inoculum with a control diet to which different substrates were added.

	Group CTRL	Group CT	Group YSE	Group CT + YSE	Pooled SEM	ANOVA *p*-value		
						Group CT	Group YSE	Group CT + YSE
At 6 h								
pH	6.37	6.37	6.36	6.36	0.01	0.955	0.436	0.822
NH_3	38.5	37.1	38.7	36.8	0.48	0.015	0.979	0.641
Putrescine	928	1030	1036	854	42.3	0.634	0.684	0.104
Cadaverine	221	225	267	140	15.7	< 0.001	0.219	< 0.001
Spermidine	32.2	32.2	29.6	34.6	1.66	0.151	0.953	0.151
Spermine	18.0	17.0	15.6	19.4	1.48	0.357	0.990	0.124
At 24 h								
pH	6.12	6.19	6.09	6.19	0.001	< 0.001	0.186	0.253
NH_3	38.2	40.8	41.3	43.8	2.77	0.366	0.287	0.980
Putrescine	1336	1326	1497	1318	52.1	0.088	0.162	0.123
Cadaverine	322	335	342	312	20.5	0.684	0.950	0.310
Spermidine	40.0	33.6	40.6	30.4	2.84	0.010	0.653	0.513
Spermine	24.6	21.0	20.0	16.2	2.38	0.140	0.066	0.967

Table 2. Mean concentrations (n = 5) of VFA [mmol/l] after 6 and 24 h of an *in vitro* incubation of canine faecal inoculum with a control diet to which different substrates were added.

	Group CTRL	Group CT	Group YSE	Group CT + YSE	Pooled SEM	ANOVA *p*-value		
						Group CT	Group YSE	Group CT + YSE
At 6 h								
Acetic acid	12.0	11.1	11.7	10.7	0.30	0.016	0.373	0.895
Propionic acid	9.86	8.62	9.40	8.95	0.26	0.019	0.837	0.232
Isobutyric acid	1.11	0.67	0.61	0.42	0.10	0.023	0.009	0.310
n-Butyric acid	4.88	4.41	4.70	4.47	0.18	0.138	0.795	0.612
Isovaleric acid	0.90	0.87	0.87	0.66	0.12	0.193	0.199	0.321
Total VFA	28.8	25.7	27.3	25.2	0.60	0.004	0.215	0.528
At 24 h								
Acetic acid	16.3	14.9	16.7	16.1	0.86	0.276	0.389	0.651
Propionic acid	14.1	11.8	15.5	13.8	0.82	0.083	0.172	0.343
Isobutyric acid	1.07	1.02	1.30	1.22	0.13	0.587	0.109	0.927
n-Butyric acid	5.84	5.78	6.09	6.26	0.41	0.893	0.399	0.771
Isovaleric acid	1.77	1.81	1.87	1.86	0.15	0.910	0.636	0.887
Total VFA	39.3	35.4	40.5	39.4	2.24	0.286	0.262	0.553

Table 3. Microbial populations [log 10 DNA copies/ml] detected by qPCR in canine faecal inoculum with a control diet to which different substrates were added. Values are the means of 5 bottles per treatment.

	Group CTRL	Group CT	Group YSE	Group CT + YSE	Pooled SEM	ANOVA *p*-value		
						Group CT	Group YSE	Group CT + YSE
At 6 h								
Lactobacillus spp.	8.06	6.35	7.11	7.06	0.54	0.132	0.837	0.157
Enterococcus spp.	7.38	6.36	9.44	8.22	0.82	0.206	0.035	0.908
E. coli	5.19	4.74	5.95	4.68	0.28	0.009	0.246	0.175
At 24 h								
Lactobacillus spp.	8.41	7.74	7.31	7.54	0.30	0.500	0.053	0.170
Enterococcus spp.	8.10	8.77	7.12	7.86	1.01	0.509	0.381	0.974
E. coli	5.33	4.93	4.84	5.37	0.33	0.857	0.942	0.193

Table 4. pH values and mean concentrations (n = 5) of ammonia [mmol/l] and biogenic amines [µmol/l] after 6 and 24 h of an in vitro incubation of feline faecal inoculum with a control diet to which different substrates were added.

	Group CTRL	Group CT	Group YSE	Group CT + YSE	Pooled SEM	ANOVA *p*-value		
						Group CT	Group YSE	Group CT + YSE
At 6 h								
pH	6.28	6.28	6.24	6.26	0.01	0.377	0.021	0.372
NH_3	33.4	34.9	34.0	33.5	0.38	0.571	0.664	0.233
Putrescine	187	200	190	179	7.74	0.860	0.188	0.074
Cadaverine	243	245	181	196	21.3	0.689	0.017	0.774
Spermidine	29.0	30.8	32.1	30.4	1.99	0.795	0.641	0.503
Spermine	5.60	5.50	5.00	4.04	0.52	0.355	0.083	0.451
At 24 h								
pH	6.10	6.14	6.05	6.17	0.01	< 0.0001	0.344	0.004
NH_3	52.2	50.9	48.7	52.0	1.68	0.777	0.744	0.536
Putrescine	303	379	373	304	23.8	0.872	0.924	0.007
Cadaverine	265	235	211	212	9.77	0.142	<0.001	0.132
Spermidine	32.0	28.4	27.4	34.4	2.68	0.534	0.797	0.065
Spermine	6.38	7.46	9.40	8.88	0.99	0.781	0.040	0.432

Table 5. Mean concentrations (n = 5) of VFA [mmol/l] after 6 and 24 h of an *in vitro* incubation of feline faecal inoculum with a control diet to which different substrates were added.

	Group CTRL	Group CT	Group YSE	Group CT + YSE	Pooled SEM	ANOVA *p*-value		
						Group CT	Group YSE	Group CT + YSE
At 6 h								
Acetic acid	14.8	13.3	12.7	12.1	0.34	0.082	0.010	0.385
Propionic acid	7.09	6.51	7.16	5.99	0.18	0.010	0.446	0.335
Isobutyric acid	0.53	0.39	0.51	0.53	0.05	0.583	0.602	0.498
n-Butyric acid	7.54	7.96	7.58	7.49	0.16	0.666	0.558	0.489
Isovaleric acid	0.63	0.65	0.62	0.68	0.04	0.646	0.894	0.788
n-Valeric acid	0.38	0.32	0.25	0.24	0.02	0.348	0.006	0.455
Total VFA	31.0	29.1	28.8	27.1	0.55	0.092	0.052	0.942
At 24 h								
Acetic acid	24.2	22.4	23.6	21.7	0.44	0.044	0.451	0.961
Propionic acid	12.7	10.6	12.8	10.0	0.34	<0.0001	0.681	0.393
Isobutyric acid	1.21	1.21	1.11	1.10	0.05	0.974	0.383	0.987
n-Butyric acid	10.1	9.68	9.86	9.29	0.14	0.095	0.292	0.718
Isovaleric acid	2.01	1.95	2.26	1.80	0.07	0.039	0.686	0.108
n-Valeric acid	2.29	3.03	2.60	3.12	0.19	0.109	0.599	0.774
Total VFA	52.5	48.9	52.3	47.1	0.75	0.002	0.422	0.497

Table 6. Microbial populations [log 10 DNA copies/ml] detected by qPCR in feline faecal inoculum with a control diet to which different substrates were added.

Values are the means of 5 bottles per treatment.

	Group CTRL	Group CT	Group YSE	Group CT + YSE	Pooled SEM	ANOVA *p*-value		
						Group CT	Group YSE	Group CT + YSE
At 6 h								
Lactobacillus spp.	8.63	7.67	7.87	7.83	0.17	0.153	0.368	0.181
Enterococcus spp.	6.13	7.82	9.42	3.87	0.73	0.105	0.769	0.008
Bifidobacterium spp.	2.70	1.51	1.09	1.60	0.28	0.154	0.160	0.119
E. coli	8.26	7.21	7.00	7.29	0.21	0.311	0.132	0.092
At 24 h								
Lactobacillus spp.	7.18	6.46	6.65	6.27	0.17	0.122	0.294	0.611
Enterococcus spp.	7.53	7.84	5.66	7.64	0.48	0.245	0.290	0.388
Bifidobacterium spp.	2.39	1.39	1.46	1.16	0.26	0.228	0.278	0.512
E. coli	6.32	5.53	5.62	5.32	0.23	0.259	0.335	0.603

Figure 1. PCA score plot of pH, bacterial metabolites and microbial populations on canine faecal inoculum

Figure 2. PCA score plot of pH, bacterial metabolites and microbial populations on feline faecal inoculum